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Use of doubled haploid technology for development of stable drought tolerant bread wheat (*Triticum aestivum* L.) transgenics

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Summary

Anther culture–derived haploid embryos were used as explants for *Agrobacterium*-mediated genetic transformation of bread wheat (*Triticum aestivum* L. cv CPAN1676) using barley *HVA1* gene for drought tolerance. Regenerated plantlets were checked for transgene integration in T_0 generation, and positive transgenic haploid plants were doubled by colchicine treatment. Stable transgenic doubled haploid plants were obtained, and transgene expression was monitored till T_4 generation, and no transgene silencing was observed over the generations. Doubled haploid transgenic plants have faster seed germination and seedling establishment and show better drought tolerance in comparison with nontransgenic, doubled haploid plants, as measured by per cent germination, seedling growth and biomass accumulation. Physiological evaluation for abiotic stress by assessing nitrate reductase enzyme activity and plant yield under post-anthesis water limitation revealed a better tolerance of the transgenics over the wild type. This is the first report on the production of double haploid transgenic wheat through anther culture technique in a commercial cultivar for a desirable trait. This method would also be useful in functional genomics of wheat and other allopolyploids of agronomic importance.

Keywords: drought, doubled haploid, *HVA1*, transgenics, wheat.

Introduction

Wheat is a cereal crop and a staple food source for billions of people worldwide. It is thus understandable that wheat has been a prime target for improvement of agronomic characteristics via genetic engineering. Genetic transformation methods offer an attractive alternative to conventional breeding programmes allowing specific traits to be transferred into selected genotypes without adversely affecting their desirable genetic makeup (Vishnudasan *et al.*, 2005; Bhalla *et al.*, 2006; Khurana *et al.*, 2008a). Wheat transformation is a lengthy process, and for most introduced genes, it is essential to produce homozygous lines of transgenic plants to adequately characterize phenotypic effects. The identification process requires screening to separate homozygous from heterozygous plants in the T_1 and T_2 generations and is labour intensive. Anther culture is a useful tool for the rapid generation of haploid plants for use in plant breeding programmes and genetic studies and also offers a means for functional genomics analysis when coupled with transgenic technology. Alternative methods for the production of haploids include isolated microspore culture (Jähne and Lörz, 1995) or chromosome elimination by pollination with an incompatible male donor (Zhang *et al.*, 1996). In comparison with these methods, the culture of anthers is simpler, less labour intensive and requires minimal facilities (Massiah *et al.*, 2001).

During anther culture, microspore development is switched from its natural gametophytic pathway to a sporophytic pathway, thus making it possible to generate homozygous plants from single cells. Anther culture has been used successfully to regenerate fertile, nontransgenic wheat (Chu *et al.*, 1990; He *et al.*, 1993; Orshinsky and Sadasivaiah, 1994; Bruins and Snijders, 1995; Jähne and Lörz, 1995). Success of the procedure is dependent on culture medium composition (Trottier *et al.*, 1993; Evans and Batty, 1994) and growth conditions during anther

pretreatment, callus formation and plantlet regeneration (Orshinsky and Sadasivaiah, 1997; Stober and Hess, 1997). Colchicine is incorporated during isolated microspore and anther culture to enable chromosomal doubling for the production of homozygous dihaploids (Barnabás *et al.*, 1991; Hansen and Andersen, 1998; Ahmed *et al.*, 1999). Besides breeding programmes, haploids are useful in research areas such as mutation studies, gene mapping, functional genomics and as a target for transformation (Dunwell, 2010). During transformation haploid embryos may be produced and used as targets for bombardment procedures or cocultivated with *Agrobacterium* to obtain transgenic doubled haploids (DH) that are homozygous for the introduced transgene(s) (Kasha and Maluszynski, 2003). However, only a few studies have been undertaken towards producing transgenic haploid plants in wheat (Brisibe *et al.*, 2000; Folling and Olesen, 2001; Massiah *et al.*, 2001; Gharanjik *et al.*, 2008), and none of these studies report successful production of transgenic DH plants.

Agrobacterium-mediated transformation has several desirable features over direct DNA delivery, such as introduction of fewer copies of genes and a higher probability of recovering single copy transgenic plants, higher coexpression of introduced genes, the ability to transfer large segments of DNA with minimal rearrangements and lower cost of experimentation (Hiei *et al.*, 1994; Gheysen *et al.*, 1998; Hansen and Wright, 1999; Shibata and Liu, 2000; Jones *et al.*, 2005). Keeping this in mind, the present work was initiated to produce transgenic DH plants in wheat through anther culture and *Agrobacterium*-mediated transformation, in commercial Indian bread wheat varieties. We show that the *HVA1* transgene is stably integrated and expressed over multiple generations and that the transgenic plants have higher tolerance to simulated water stress.

Results and discussion

Induction of androgenesis

To rapidly generate transgenic DH plants of commercial cultivars, an anther culture–based approach for haploid embryo induction and *Agrobacterium*-mediated transformation was adopted. The protocol utilized a liquid culture phase for embryo induction, followed by *Agrobacterium*-mediated transformation and plantlet regeneration on gelled medium. Of the six commercial Indian bread wheat genotypes tested for haploid induction (*Triticum aestivum* cv.UP2338, PBW343, HD2428, HD2329, HD2687 and CPAN1676), only CPAN1676 responded. This may be attributed to the considerable genotypic variation in response to *in vitro* tissue culture in general and to androgenesis in particular in wheat (Moiene and Sarrafi, 1995; Chauhan *et al.*, 2007). Embryo-like structures (ELS) were visible as white beads in the 24-well plates over liquid medium after 5–6 weeks of culture (Figure 1b). These ELS germinated into plantlets upon transfer to 190-2R regeneration medium (Figure 1c,d). The presence of several albino plantlets was also observed which is quite common during cereal anther culture. The haploid nature of the regenerants was confirmed by root tip cytology and stomatal guard cell length determination (Figure 1g,h). Root squash preparation revealed the presence of the haploid chromosome set of $n = 21$ (Figure 1i). The average haploid stomata guard cell length was 33 μm (Figure 1g), while that for the hexaploid control was 65 μm (Figure 1h). The frequency of chromosome doubling by colchicine treatment was 48% (Table 1). Nearly half of the plants died subsequent to colchicine treatment, which may be attributed to chromosomal abnormalities but was not examined in details.

Transformation of haploid ELS

Agrobacterium-mediated genetic transformation was undertaken using haploid embryos as the target tissue. This tissue was found to be extremely responsive to T-DNA delivery as indicated by transient GUS assay (Figure 1e,f). Holiloglu *et al.* (2004) also reported wheat anther culture-derived embryo transformation via electroporation; however, only albino plants were regenerated on the selection medium. In the present study, *Agrobacterium*-mediated transformation was attempted and regeneration of stable green haploid plants obtained. The regenerating ELS showed high transient GUS activity (Figure 1 and Table 1), and GUS activity was also seen in young leaves of regenerating haploid seedling (Figure 1f). Plants were grown in potted soil, and genomic DNA extracted from the leaf tissue. PCR was undertaken for the full-length ORF for the *NPTII*, *HVA1* and *GUS* genes. PCR analyses of the T_1 transgenic DH plants revealed the presence of transgene in the treated but not in control plants (data not shown) and also verified lack of segregation (no nontransgenic segregants) as expected for the progeny of DH plants. The homozygosity was achieved in the first generation (data not shown), and future generations were meant only for sufficient seed production for stress tolerance analysis. In a previous study, Massiah *et al.* (2001) found that transgene inheritance in

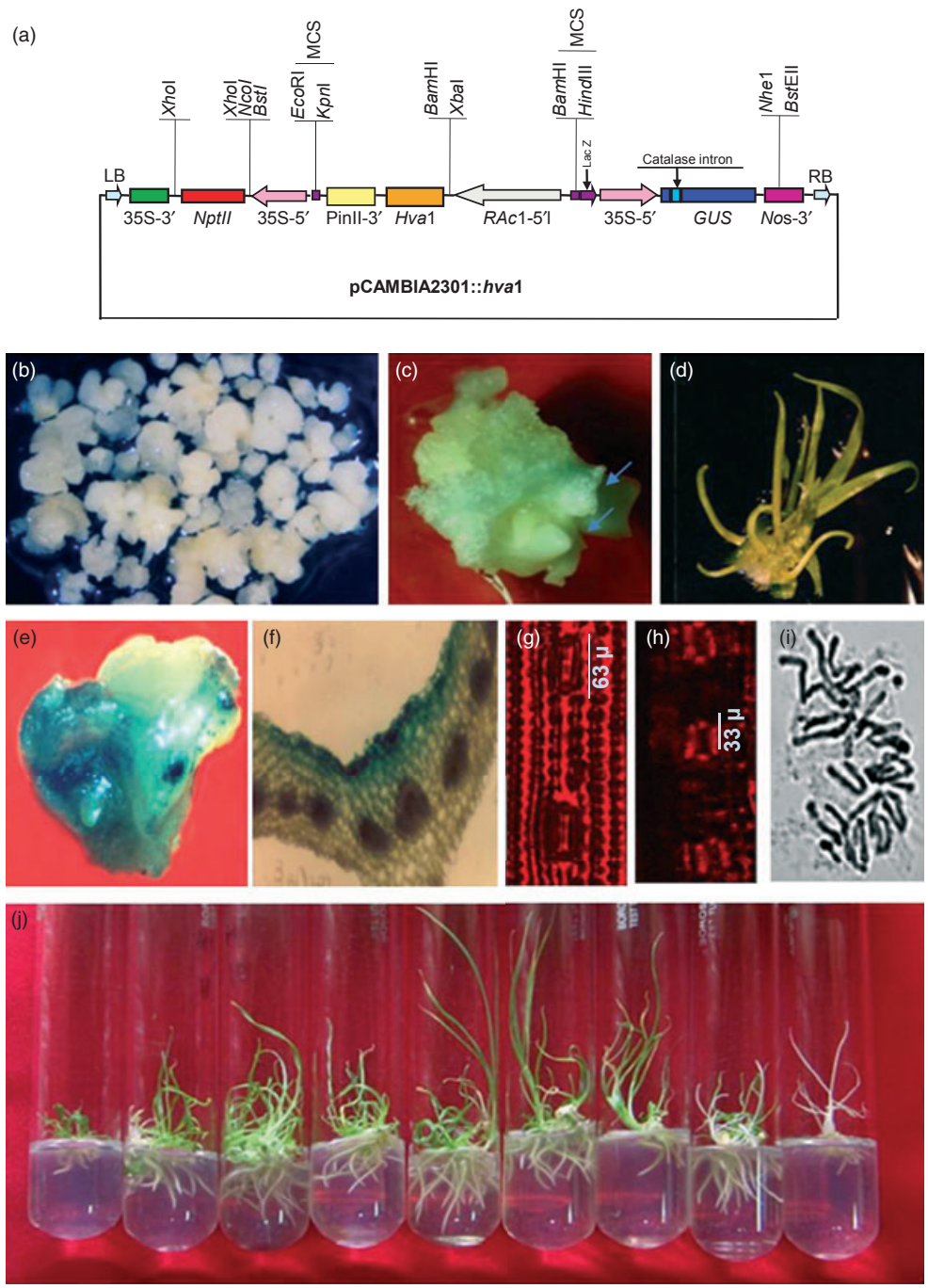

Figure 1 (a) Vector map of pCAMBIA2301:*hva1* (b–d) Anther culture–derived embryo induction and conversion to plantlets in *Triticum aestivum* cv. CPAN1676, (e-f) GUS histochemical assay in transgenic embryo and first leaf, (g–i) Confirmation of ploidy by stomata guard cell length (400×) and by root tip squash, (j) Transgenic haploid plantlets growing on selection.

haploid plants followed the pattern of normal seed–derived transgenic plants. T_1 seed–derived plants were also checked for the integration of the transgene (*HVA1*) by Southern hybridization.

Genomic DNA (10–20 µg) of putative transformants was digested overnight at 37 °C with *XhoI* (for *NPTII*), *XbaI* (for *GUS*) or *EcoRI* and *BamHI* (for *HVA1*) restriction enzymes which release *NPTII, GUS* and *HVA1* fragments, respectively, from the vector. There is a single site for *XbaI* in the MCS of pCAMBIA2301:HVA1 T-DNA, which was used for copy number detection. Southern analysis, using *NPTII* and GUS as probes, revealed that these DH trans-

Table 1 Anther culture and transformation response of *Triticum aestivum* cultivar CPAN1676

Total Anthers cultured	2000
Haploid embryos transformed	210
Percentage of transformation based on transient GUS expression	100%
Total kanamycin-resistant T_0 haploid plants	21
Total doubled haploid (DH) plantlets from kanamycin-resistant T_0 plants	10
Total DH kanamycin-resistant plants setting seed	9
Total independent confirmed transgenic DH events based on T_1 molecular analysis	5

genic plants each had a single copy of the transgene as digestion with either *XhoI* or *XbaI* revealed a single band for each plant examined (Figure 2a,b). Further, Southern analysis with the GUS gene probe hybridized to XbaI-digested genomic DNA showed that these plants represented at least five independent transgenic events (Figure 2b). However, Southern analysis using *HVA1* as a probe revealed multiple bands in both WT and transgenic DH lines, indicating a cross-reactivity of barley *HVA1* with highly similar genes of the wheat *LEA* family (Supplementary Figure S1). This is expected as there is nearly 80%–90% homology in the LEA superfamily in cereals. Nevertheless, specific expression of the introduced *HVA1 gene* was confirmed both at the transcript and at the protein level by RT-PCR and Western analysis, respectively. RT-PCR for both *NPTII* and *HVA1* was performed on T_3 plants (data not shown). It was found that both these gene transcripts could be detected in all the plants subjected to RT-PCR. *HVA1* transcripts as well as the protein levels could be detected in young seedlings of all the nine transgenic DH lines tested in the T_4 generation (Figure 3) and not in the wild-type (WT) control.

Evaluation of DH transgenic plants

Upon germination of T4 seeds, faster germination and seedling establishment in transgenic DH lines were observed in comparison with WT–DH (Figure 4). The coleoptile and roots of transgenic DH lines emerged at least 24 h earlier than that of WT–DH lines and maintained a rapid pace of growth subsequently. After 5 days, the first leaf was almost fully expanded in the transgenic lines, while in the WT, coleoptile was seen with the half emerged first leaf. Tolerance of transgenic DH and WT–DH against simulated water stress was also checked. It was found that transgenic plants had not only a faster germination (Figure 4) but also longer roots and shoots (Figure 5a) and higher nitrate reductase (NR) activity (Figure 5b) at all tested concentrations of mannitol. DH plants were relatively unaffected by simulated water stress even up to 300 mM mannitol, while WT plants showed reduced growth and development (Figure 4). At 400 mM mannitol, WT–DH seeds did not germinate at all, while transgenic seeds not only germinated but also had

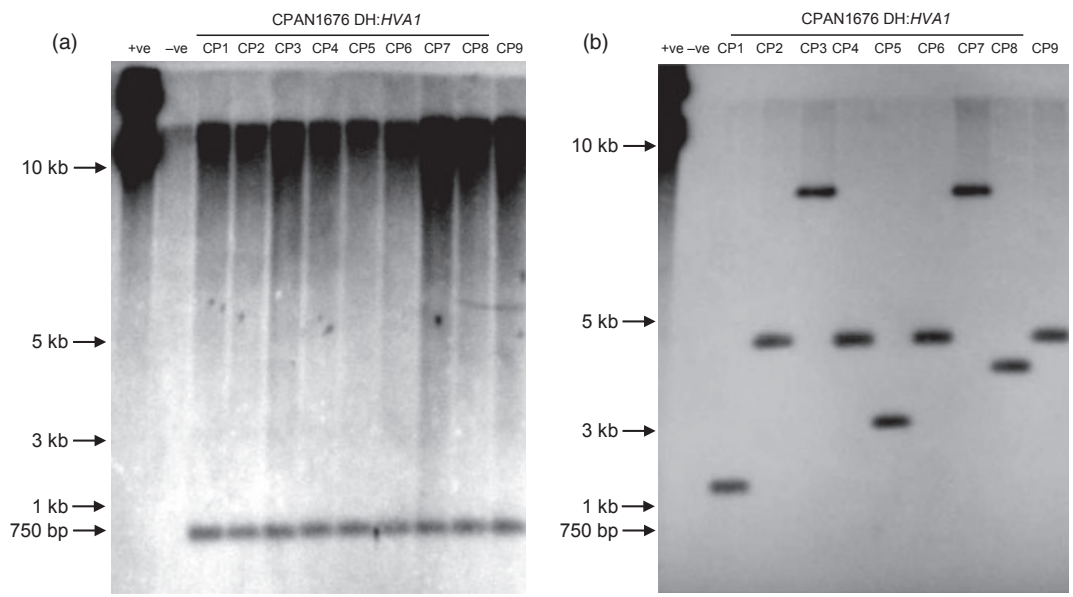

Figure 2 Southern analyses of transgenics by *NPTII* (a) and *GUS* gene (b) in T_2 doubled haploid (DH) plants. (a) Genomic DNA digested with *XhoI* (b) Genomic DNA digested with *XbaI.*

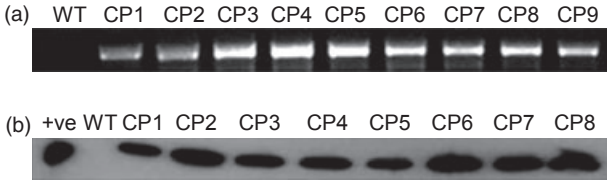

Figure 3 Expression of *HVA1* gene in 10- day-old seedlings of T_4 transgenic doubled haploid (DH) plants of *Triticum aestivum* cultivar CPAN1676 (a) RT-PCR, and (b) Western analysis using anti HVA1 antibodies.

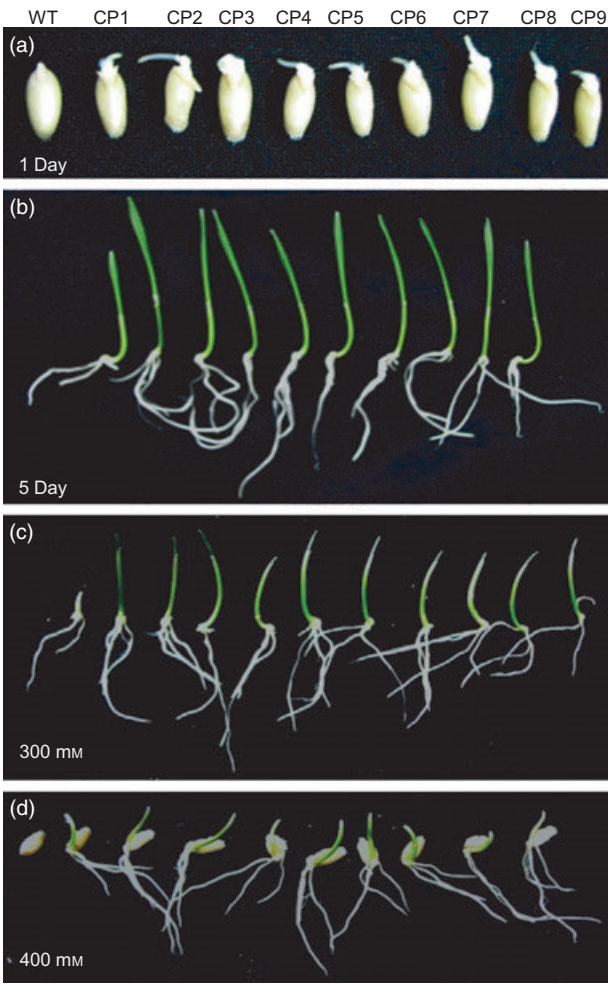

Figure 4 Performance of WT and different T_4 transgenic doubled haploid (DH) lines during germination at different time points (a and b) and under simulated drought stress by mannitol (c and d).

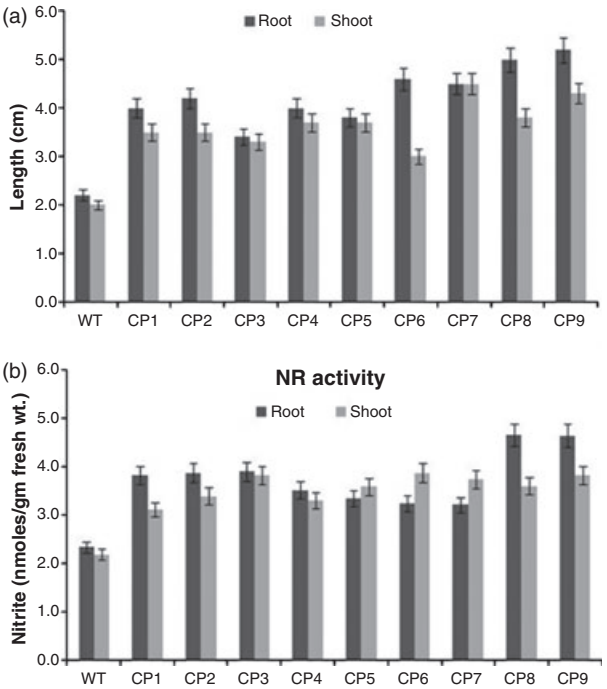

Figure 5 Effect of simulated drought stress by 200 mM mannitol in WT and different T_4 transgenic doubled haploid (DH) lines on root and shoot length (a), and nitrate reductase activity (b) in root and shoot tissues of 10-day-old seedlings ($P = 0.05$).

appreciable root length. Average root–shoot length and *in vivo* NR activity measured at 200 mM mannitol revealed that transgenic plants had not only better seedling growth but also higher NR enzyme activity in both roots and shoots (Figure 5). Previous results indicated the potential of *LEA* genes for the genetic improvement of plants for water-limiting environments (for review see Khurana *et al.*, 2008b). *HVA1* is a group 3 *LEA* gene and has been used for transformation for enhancing drought tolerance in both monocots and dicot plants. (Xu *et al.*, 1996; Su *et al.*, 1998; Sivamani *et al.*, 2000; Cheng *et al.*, 2002; Lal *et al.*, 2008). Babu *et al.* (2004) reported that transgenic rice plants overexpressing *HVA1* had higher membrane stability and better plant growth in terms of root and shoot weight under drought stress conditions. We also found that transgenic wheat DH plants overexpressing *HVA1* had much better root and shoot growth even under 400 mM of mannitol. Although, the exact mode of action of LEA proteins is not clear, several possibilities exist for their function. Reports indicate a possible role of *LEA* proteins in dehydration tolerance through maintenance of protein and/or membrane structure, sequestration of ions, binding of water and function as a chemical chaperon (Dure, 1993; Goday *et al.*, 1994; Close, 1996). Group 3 LEA proteins characteristically possess an 11-mer amino acid motif with consensus sequence TAQAAKEKAGE (Dure, 1993). In HVA1, this motif is repeated as many as nine times forming an alpha helical dimer suitable for accommodating positively and negatively charged ions and thus providing a putative function in ion sequestration (Lal *et al.*, 2008). Nitrate reductase is a water stress–sensitive

enzyme (Foyer *et al.*, 1998; Garg *et al.*, 2001; Correia *et al.*, 2005), and its activity reflects nutrient uptake capacity. Under drought stress, NR activity was severely reduced in leaf tissues of wheat plants (Fresneau *et al.*, 2007). We observed that transgenic DH wheat plants showed almost double NR activity under simulated water stress conditions over the WT. Thus, overexpression of *HVA 1* may lead to a better protein and membrane integrity leading to better growth under water-limiting conditions, and because majority of cellular NR is membrane bound, we hypothesize that HVA1 protects NR activity through the stabilization of membrane integrity under stress conditions.

Photosystem II plays a key role in response to environmental changes (Baker, 1991). Efficiency of PSII can be measured by chlorophyll fluorescence, thereby indirectly measuring photosynthetic capacity of plants. Photoinhibition in the flag leaf is reflected by decreased Fv/Fm owing to post-anthesis water stress. At field capacity, the soil moisture content was around 50% in all the pots; however, after 10 days of water limitation, the treatment pots had soil moisture around 15% (data not shown). We found a drastic decrease in PSII activity in flag leaves of WT plants as measured by Fv/Fm and ETR, while transgenic DH plants fared better in both PSII activity and relative water content (RWC) of the flag leaf (Figure 6a).

Post-anthesis drought stress is known to reduce wheat yield (Stone and Nicolas, 1994, 1995). Number and weight of kernels per spike along with 1000 grain weight have been suggested as important selection criteria for selecting drought tolerant cultivars (Shipler and Blum, 1991; Dencic *et al.*, 2000). In the present study, we found that transgenic double haploid plants performs much better with respect to these parameters, especially seed weight per spike than WT plants (Figure 6b).

It takes several years from an initial transformation event to the generation of a variety having superior agronomic traits for commercial use. Production of transgenics and screening for desirable phenotypes followed by generation of homozygous lines is labour and resource intensive. In the present investigation, we have shown the practical feasibility of generating stable transgenics for a desirable trait and subsequent homozygous line generation in a single crop cycle saving both time and resources. Plants produced are stable for transgene inheritance and expression. Although the number of transgenic DH plants is still less, we get single-copy transgenics. However, this can be finally ascertained with getting more transgenic plants representing more events. Moreover, there is a need to work towards a comparatively genotype-independent, anther culture protocol in wheat.

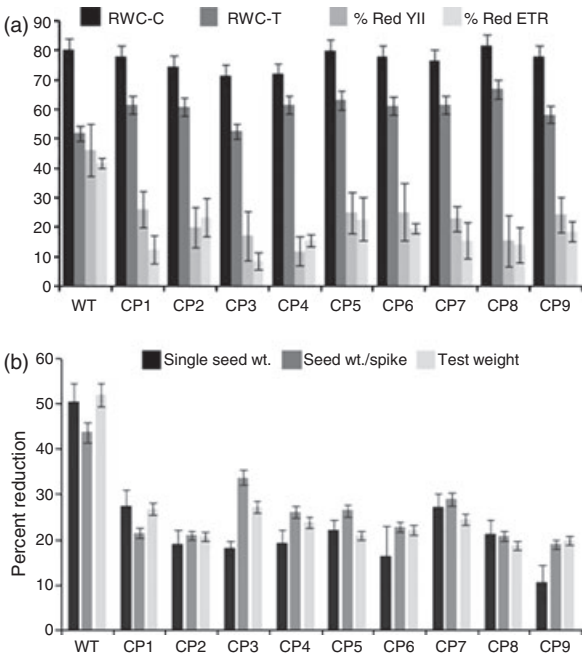

Figure 6 (a) Physiological performance of WT and T_4 transgenic doubled haploid (DH) plants under post-anthesis water limitation. (RWC-C and RWC-T represents relative water content in control and treated plants, respectively; *C* = 50% soil moisture, *T* = 15% soil moisture). (b) Yield performance of WT and T_4 transgenic DH plants under post-anthesis water limitation ($P = 0.05$).

Experimental procedures

Donor plants and growth conditions

In the present investigation, the wheat anther culture protocol as described by Pauk *et al.* (2003) was followed. Initially, six commercial Indian genotypes (i.e. UP2338, PBW343, HD2428, HD2329, HD2687 and CPAN1676) were tested for haploid induction along with Chinese Spring. Only CPAN1676 responded well in terms of haploid embryo induction. We have previously shown that this variety has excellent *in vitro* regeneration from three different explants tissues (Chauhan *et al.*, 2007). Therefore, we used this cultivar for haploid embryo induction and transformation studies through anther culture. For anther culture, wheat plants were raised in the departmental garden during the normal crop season (November–April) from 2004 to 2008.

Pretreatments

The donor tillers containing microspores at the late uninucleate stage were cut between the second and third nodes, and the selected tillers with the spikes in the penultimate leaf sheath were kept in dark in Erlenmeyer flasks with tap water, covered by plastic bags to maintain high humidity for cold pretreatment at

4 °C for 2 weeks. Before anther isolation, the microspore developmental stage was checked microscopically. Spikes containing early- and mid-uninucleate microspores were surface-sterilized with 2% NaOCl containing two drops of Tween-80 for 20 min on a shaker and then rinsed three times with sterile distilled water. Anthers were excised onto a thin layer of liquid P-4MF medium (Pauk *et al.*, 2003), in a 24-well plate containing 2 mL of media per well. Twenty anthers along with 1–2 ovaries were plated per well. These 24-well plates were then incubated in the dark at high humidity (80%) for 3 days at 32 °C (heat treatment). After 3 days, these plates were transferred to a growth room maintained at 28 °C for 5–6 weeks, in dark. Embryo-like structures (ELS) were visible as white beads in the liquid medium 4–6 weeks after isolation (Figure 1a). When the size of ELS was about 1.5 mm, these were used for *Agrobacterium*-mediated transformation.

Transformation of haploid embryos

Plasmid pBY520 containing *HVA1* cDNA with *Act1* promoter and potato *pinII* terminator (Hong *et al.*, 1988) was obtained from Ray Wu (Cornell University, NY, USA). Region containing *HVA1* with its promoter and terminator was mobilized into pCAMBIA2301 at Sma1 site to generate pCAMBIA2301:*hva1* (Figure 1a). *Agrobacterium*-mediated transformation with pCAMBIA2301:*hva1* was undertaken according to Patnaik *et al.* (2006). The axenically isolated haploid embryos of CPAN1676 were inoculated with *Agrobacterium* [LBA 4404 (pCAMBIA2301:*HVA1*)] at $\sim 5 \times 10^8$–1×10^9 cells/mL density along with 200 μM acetosyringone (Sigma-Aldrich, St. Louis, US) for 3 h, after which the embryos were placed on Petri plates containing cocultivation medium. After 3 days of co-cultivation on MS medium containing 2 mg/L 2,4-D and 200 μM acetosyringone, the embryos were washed with half-strength liquid MS and then assayed histochemically for GUS expression (Jefferson *et al.*, 1987). To assay for stable expression, we incubated shoots and leaf fragments overnight from regenerating plantlets at 37 °C and, if necessary, for further 1–2 days at the same incubation temperature.

Regeneration of haploid plants, ploidy determination and chromosome doubling

After washing, the *Agrobacterium* cocultivated haploid embryos were incubated on regeneration medium 190-2R (Pauk *et al.*, 2003) supplemented with kanamycin (50 mg/L) for 3–4 weeks at 24–26 °C, light intensity 100–125 μmol/(m^2 s), 16/8 h photoperiod provided by cool-white fluorescent tubes. After 1 month of incubation, both green and albino plantlets emerged. These plantlets were then transferred to individual culture tubes containing 190-2R medium for rooting and proliferation. After about 2–3 weeks of growth on selection medium, the rooted green plantlets were transferred to earthen pots containing a mixture of soilrite (Kel Perlite, Bangalore, India) and soil (1 : 1) and grown to maturity in a growth chamber (Conviron, Control Environments Limited, Winnipeg, Canada) operating at 21 °C, 16 h light at 100–125 μmol/(m^2 s) and 70% relative humidity. The plants were supplied with a liquid medium recommended for the growth of wheat plantlets (Lee *et al.*, 1989), and the well-growing plantlets used for ploidy level determinations by the measurement of stomata guard cell length in 10- mm distal leaf segments. Chlorophyll was extracted in 70% alcohol, and the length of stomata guard cells measured by a confocal microscope (Leica, Wetzlar, Germany). Root tip squashes were also undertaken to determine the ploidy of the plants by acetocarmine staining.

For chromosome doubling, colchicine (0.2%) was prepared in a 2% DMSO solution. The haploid plantlets at 4–5 leaf stage were washed under running tap water to remove the soil, and the roots trimmed back by about 2 cm. The plantlets were then treated in the colchicine solution for 5 h in glass vials. Colchicine treatment was followed by an overnight washing under tap water. The shoots were then trimmed back to about 10–12 cm (depending on individuals) before transplanting into pots. The treated plantlets were then transferred to the growth chambers under 16 h light at 100–125 μmol/(m^2 s) and 21 °C temperature and grown till maturity.

Southern analysis of the putative transformants

Digested genomic DNA (20 μg) was electrophoresed on a 0.8% w/v agarose gel and capillary blotted on Hybond-N membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) using 20× SSC, and membranes were cross-linked with the help of a UV cross-linker (Amersham Pharmacia Biotech) and used for Southern hybridization. For probe preparation, *NPTII*, *GUS* and *HVA1* fragments were amplified by PCR from pCAMBIA2301:*HVA1* plasmid. These fragments were radiolabelled using Megaprime DNA Labelling kit (Amersham International Inc, UK) with α-^{32}P ATP (specific activity 3000 Ci/mmol BRIT, India) as per manufacturer's specifications.

Sequence analysis and phylogenetic relationships

Nucleotide and the deduced amino acid sequences of *HVA1* were searched for their homology with the previously existing wheat (*T. aestivum*) sequences in the NCBI database using the BlastN and BlastP programs (Altschul *et al.*, 1997). Phylogenetic tree was constructed using CLUSTAL X programme from available wheat *LEA* protein sequences retrieved from the NCBI database, by neighbourhood joining method.

RT-PCR analysis

Total RNA was isolated using the RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including on-column DNase I treatment to remove genomic DNA contamination. For expression analysis of *HVA1* in different transgenic lines, a two-step RT-PCR was employed. Two micrograms of RNA from each sample was used to synthesize the first-strand cDNA using the SuperScriptIII first strand cDNA synthesis kit (Life Technologies, California, US). RNA was taken in a 20-μL reaction volume containing 1× reaction buffer, 1 μL of oligo dT_{20} primer (50 μM), 2.5 mM each of dCTP, dTTP, dGTP, dATP and 200 units of reverse transcriptase SuperscriptIII. After incubation at 45 °C for 1 h, RNA was removed by incubating with RNase H at 37 °C

for 20 min. After RNase H treatment, 80 μL MQ water was added to each tube, and 1 μL of cDNA template was added in each PCR with *HVA1* gene-specific primers (*HVA F*-5′-ATG GCC TCC AAC CAG AAC C-3′ and *HVA R*-5′-CTA GTG ATT CCT GGT GGT G-3′). PCR was conducted with the following program using Taq DNA polymerase (Roche, Mannheim, Germany): initial denaturation at 94 °C for 5 min, followed with 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min with 30 cycles. The PCR products were checked on 1.2% agarose gel in 1× TAE buffer with EtBr.

Production of polyclonal antibodies against *HVA1*

For production of polyclonal antibodies, *HVA1* ORF without the stop codon was amplified with ExHVA1EcoRI-F (5′-GAA TTC ATG GCC TCC AAC CAG AAC C-3′) and ExHVA1XhoI-R (5′-CTC GAG CTA GTG ATT CCT GGT GGT G-3′) primers from pCAMBIA2301:*HVA1* plasmid and fused with 6His tag of pET28a vector (Novagen) in *EcoRI* and *XhoI* sites. The fusion protein was induced and purified with Ni-NTA agarose beads as per the manufacturer instruction (Qiagen). The purified protein was given to M/s. Bangalore Genei (India) for antibody production in rabbits.

Protein extraction, gel electrophoresis and Western analyses

For extraction of steady-state soluble proteins, samples were homogenized in liquid N_2 to a fine powder with the help of a pestle and mortar. The ground powder was suspended in a buffer consisting of 30 mM Tris–HCl (pH 8.5), 1 mM ascorbic acid, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Zivy *et al.*,1983). Samples containing 50 μg of protein were loaded onto a 12.5% SDS polyacrylamide gel and run at 25 mA per gel. For immunoblot analyses, proteins were transferred to Hybond P membranes (Amersham Biosciences, UK) using a gel blotter. Membranes were blocked with 5% (w/v) non-fat milk powder in TBS/T (137 mM NaCl, 20 mM Tris-HCl, pH 7.6, and 0.5% (v/v) Tween-20 and incubated for 3 h with anti-HVA1 antibodies. A chemiluminescence detection kit (Amersham Biosciences) was used for HVA1 protein detection.

Simulated drought stress treatment

T_4 transgenic DH seeds were assessed for drought stress tolerance along with WT seeds. For simulating drought stress, seeds were germinated on cotton pads soaked with water and supplemented with varying concentrations of mannitol in plastic trays. Trays were kept in a growth room maintained at 20 °C in a 16 : 8 light : dark photoperiod. Data related to per cent germination, root and shoot length was recorded at various time points.

For mature plant drought stress response, T_4 transgenic DH plants along with WT–DH plants were grown in earthen pots in a randomized block design in the growth chamber, with a density of five plants per pot. Post-anthesis drought stress was provided to whole plants by withholding the water application at the spike emergence stage. In flag leaf, data related to RWC and photosynthetic yield (chlorophyll florescence by Junior PAM, Walz, Germany) was taken as described previously (Lal *et al.*, 2008) under both control and water-stressed conditions. Nitrate reductase (NR) activity was measured as described by Sairam and Srivastava (2000). Yield related data was scored after plant maturity.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Analysis of T_2 transgenic doubled haploid (DH) plants of *Triticum aestivum* cv CPAN1676.

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